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A quantitative study of adipokinetic hormone of the firebug, *Pyrrhocoris apterus.*

Graham J. Goldsworthy [a,*], Dalibor Kodrík [b], Robert Comley [c] and Mary Lightfoot[a]

[a] Department of Biology, Birkbeck College, University of London, Malet Street, London, WC1E 7HX, UK
[b] Institute of Entomology, Czech Academy of Sciences, Branišovská 31, CZ-370 05 České Budějovice, Czech Republic
[c]GlaxoSmithKline, Addenbrooke's Centre for Clinical Investigation (ACCI), Addenbrooke's Hospital, Hills Road, Cambridge, UK

- Corresponding author: Prof. Graham J. Goldsworthy, Department of Biology, Birkbeck College, University of London, Malet Street, London, WC1E 7HX, United Kingdom

Tel. No.: 044 (0)20 7631 6352 Fax No.: 044 (0)20 7631 6246; E-mail: g.goldsworthy@bbk.ac.uk

Abstract
The development of an enzyme-linked immunoassay (ELISA) for the adipokinetic neuropeptide hormone, *Pya*-AKH, from the firebug *Pyrrhocoris apterus* L. is described. The ELISA measures as little as 20 fmol of *Pya*-AKH. Tested against a range of synthetic peptides, the assay has a high sensitivity for peptides containing the C-terminal motif FTPNWamide. The amounts of *Pya*-AKH in the brain, corpora cardiaca, suboesophageal ganglia, and fused thoracic and abdominal ganglionic mass are very small, with only the corpora cardiaca containing appreciable levels of hormone (*c*. 4 pmol per bug). Preliminary estimates of the persistence of the hormone in the haemolymph are consistent with values determined for AKHs in other insects, and suggest that the *Pya*-AKH has a rapid turnover with a half-life of *c*. 18 min. Measurements of circulating titres of AKH in *Pyrrhocoris* are only possible in the ELISA described here by using pooled samples of haemolymph, and after preliminary clean-up of the haemolymph samples. The titre of *Pya*-AKH in resting reproductive female *Pyrrhocoris* is *c*. 1.14 fmol/μl.

Keywords: Adipokinetic hormone; ELISA; *Pyrrhocoris apterus*; CNS; haemolymph

Introduction

Neuropeptides belonging to the adipokinetic hormone (AKH) family have been identified in almost all insect orders (Gäde *et al*., 1997) including the Heteroptera, in which two octapeptide members, *Pya*-AKH and *Pea*-CAH-II, have been identified recently (Kodrík *et al*., 2000; 2002a). In the firebug, *Pyrrhocoris apterus* L., both *Pya*-AKH and *Pea*-CAH-II mobilise diacylglycerols into the haemolymph and at least *Pya*-AKH stimulates locomotory activity (Kodrík *et al*., 2000; 2002a). While there is a great deal of information available concerning the likely physiological functions of AKHs in insects that fly, for example moths, locusts, and beetles (see Ziegler and Schulz, 1986ab; Ziegler, 1990; Goldsworthy and Joyce, 2001; Auerswald and Gäde, 2002), many flightless insects possess AKHs whose physiological roles are largely unknown. This paper describes the development of an enzyme-linked immunoassay (ELISA) so that information about the levels of AKH in the tissues and haemolymph of *Pyrrhocoris* can be obtained. This is a first step in extending our knowledge of the behavioural and physiological roles of AKH in this flightless insect.

Materials and Methods

Experimental animals

A laboratory stock culture of the firebug *Pyrrhocoris apterus* in the Institute of Entomology was used. The origins and breeding details of the culture have been described by Socha and Šula (1996). Bugs were maintained at 26 ± 1°C in a long day LD 18:6 h photocycle, and were supplied with linden seeds and water in small glass tubes plugged with cellulose wool. Larvae and adults were kept in 0.5-l jars in mass culture (approximately 40 specimens per jar), and food and water were replenished twice a week. Reproductive females, from 7 to 10-day old, were used in all experiments.

Development of the ELISA

Rabbit antibodies were raised commercially against Cys^{1}-*Pya*-AKH (Sigma Genosys, Cambridge, UK) coupled to keyhole limpet haemocyanin using a standard protocol. Immunoglobulin was prepared at Birkbeck from the resulting antiserum by selective ammonium sulphate precipitation (Harlow and Lane, 1988 p298-299). Synthetic *Pya*-AKH and Cys^{1}-*Pya*-AKH were custom synthesised by Sigma Genosys (Cambridge, UK).

A biotinylated probe was prepared from Cys^{1}- *Pya*-AKH using Biotin Long Arm Maleimide (BLAM, Vector Laboratories, Peterborough, UK). Using the manufacturers recommended protocol, the first attempt at producing this probe was unsuccessful: within minutes of adding 5 µl of the BLAM (20 mg/ml of DMSO) solution to 200 µl of Cys^{1}- *Pya*-AKH (5 mg/ml in 100 mM phosphate buffer, pH 7), a copious white precipitate formed that would not re-dissolve in phosphate buffered saline at pH 7.4 (PBS). Its identity was not investigated further in any detail, but HPLC analysis (see below) showed that very little of the Cys^{1}- *Pya*-AKH remained in the supernatant of the reactant solution. However, by using the BLAM at a 5 times molar excess over the Cys^{1}-*Pya*-AKH, a PBS-soluble product was obtained: within 15 min of adding 12.6 µl of BLAM (1.9 mg in 20 µl of DMSO) to 0.5 mg of Cys^{1}-*Pya*-AKH dissolved in 130 µl of phosphate buffer (pH 7), almost complete conversion of the Cys^{1}- *Pya*-AKH to a single fluorescent peak of more hydrophobic product was observed in the HPLC analysis.

The biotinylated Cys^{1}- *Pya*-AKH was purified by gradient elution on a reversed phase Poros R2 column (100 x 4.6 mm) using a Waters HPLC controlled by

Millennium® software. Solvent A was 0.1% TFA (trifluoroacetic acid) in deionised water, and solvent B was 0.075% TFA in 95% CH_3CN. The gradient ran from 70 to 40% A over 12 min at a flow rate of 3 ml/min. Elution was monitored for absorbance at 208 nm and by measurement of fluorescence (λ_{Ex} 280 nm; λ_{Em} 348 nm) due to the presence of tryptophan. Cys[1]- *Pya*-AKH gave a single fluorescent peak with a retention time of 4 min in this system, whereas biotinylated Cys[1]- *Pya*-AKH had a retention time of 4.7 min and was well-resolved from its parent. This biotinylated product was collected, concentrated in a vacuum centrifuge to remove acetonitrile and TFA, and the methanol content made up to 80% for storage at -15°C until required: it was quantified in solution (see below) and is referred to as the biotinylated probe (BLAM-Cys[1]- *Pya*-AKH).

Titration of the dilution of anti-Cys[1]- *Pya*-AKH IgG against decreasing amounts of biotinylated probe suggested that optimum conditions for a competitive ELISA were a dilution of between 1:10000 and 1:5000 of the IgG and 100 fmol/well of the BLAM-Cys[1]- *Pya*-AKH. Thus the ELISA comprised pre-coating of the 96-well microtitre plates (enhanced binding Combitech Labsystems plates, Life Sciences International) overnight at 4°C with 100 µl of the IgG preparation diluted in coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6). After washing in PBS (at pH 7.4), and blocking with non-fat dried milk powder solution (5 g/100 ml PBS, containing 0.02% Tween-20), test samples in 50 µl of assay buffer (PBS, no Tween) were added to specific wells, followed by 100 fmol of the biotinylated probe contained in 50 µl of assay buffer. Competition for the binding sites on the IgG bound to the plates was for 1h at 37°C (with shaking). The wells were then washed in PBS, and 100 µl of streptavidin conjugated with horseradish peroxidase solution (Vector Laboratories, diluted 1:500 in PBS-Tween), added to each well and incubated again at 37°C with shaking for 30 min. After further washing, 100 µl of freshly prepared OPD reagent (4.25 mg ortho-phenylenediamine (Sigma) + 12.15 ml of 0.1 M citrate buffer at pH 5 + 12.85 ml of 0.2 M phosphate buffer + 1.25 µl of 30% hydrogen peroxide) added to each well and incubated in the dark at 37°C with shaking for 40 min, after which 50 µl of 0.5 M sulphuric acid were added to each well and the absorbance values determined in a microtitre plate reader at 492 nm. One row of each plate contained a dilution series of *Pya*-AKH for the construction of a competition curve: this included one well with no material competing with the probe, to determine the maximum absorbance (MAX), and one well with an excess of 10 pmol *Pya*-AKH to determine the non-specific absorbance (NSA). The percentage competition for each sample was calculated as: 100-100*(absorbance of the sample - NSA)/(MAX- NSA). Samples were usually run in triplicate on each plate.

Synthetic AKHs and their quantification

The synthetic AKHs (other than *Pya*-AKH) used in this study came from a variety of sources (see Lee *et al*., 2000). In all cases they were quantified in aqueous solution by measuring their fluorescence due to the presence of at least one tryptophan residue (λ_{Ex} 280 nm, λ_{Em} 348 nm) per molecule, and comparison with fluorescence values from known concentrations of free tryptophan.

Turnover of the Pya-AKH

Four groups of 10-day old females were used in this study. Using a Hamilton syringe, the females were injected in the thorax with 2 µl of experimental solution (dissolved in 20% methanolic saline) containing increasing amounts of *Pya*-AKH (0, 10, 20 and 80 pmol per bug). Samples of haemolymph were taken over a period of 4h

from a different bug each time from each of the groups injected with AKH: a drop of haemolymph exuding from the cut end of an antenna was collected on a piece of Parafilm M®, and 1 µl of this was taken up using a micropipettor (Eppendorf Varipipette 4810). The amount of AKH remaining in the haemolymph samples was determined using the ELISA after extraction in 80% methanol, drying, and being taken up in PBS with sonication.

Extraction of the AKH from CNS

To avoid possible losses of the peptidic material from the corpora cardiaca, and to dissect undamaged corpora cardiaca, the tissue samples were prepared of the brain alone, and of the brain with corpora cardiaca and corpora allata attached. Preparations were made also of the nerve ganglia (suboesophageal, thoracic and abdominal ganglionic mass together). All dissected tissues were from 7-10-day old females, and were stored frozen until needed. Subsequently, the tissues were extracted in 80% methanol with sonication. The supernatant of the methanolic extract was then evaporated to dryness, the residue taken up in PBS with sonication, and the AKH content determined using the ELISA described here.

Extraction of the AKH from the haemolymph

For determination of the endogenous level of AKH in haemolymph from *Pyrrhocoris* by the competitive ELISA, some pre-purification steps were essential. The total volume of haemolymph in a reproductive female was estimated to be *c*. 6 µl, and it was possible to obtain 2-3 µl from each bug. Pooled haemolymph from at least 10 bugs, but often as many as 20, was extracted in 80% methanol and the supernatant evaporated to dryness, taken up in 0.11% TFA (trifluoroacetic acid), and applied to a solid phase extraction cartridge (Sep Pak C18, Waters). The cartridge was washed with 0.11% TFA, and the fraction eluting in 60% acetonitrile evaporated to dryness and taken for HPLC analysis on a Merck-Hitachi D-6000 chromatography system using HPLC System Manager D-7000 software, at a flow rate of 2 ml/min and UV monitoring at 215 nm. The sample was fractionated on a Chromolith Performance RP-18e column (Merck, 100 x 4.6 mm) with the following programmed gradient: 0-2 min 25% B; 2-12 min 25-87% B; and 12-15 min 87% B (A = 0.11% TFA in water, B = 60% acetonitrile in 0.1% TFA). Because of the small amounts of AKH material present, eluted fractions were tested without replication in the ELISA as described above, and with an IgG dilution of 1:5000 used to coat the plates for these haemolymph fractions.

Data handling and presentation

The data for figures 1 and 3 were plotted in FigP (Biosoft Ltd.). In the competition studies, the amount of peptide that was required in each well to displace half of the probe was determined from Hill plots (FigP, Biosoft Ltd) of the percentage competition against amount of peptide. For *Pya*-AKH this was on average 113 fmol, and data for each peptide (in fmol) were normalised by dividing by 1.13 to give a relative recognition value (see Table 1). The data for Figures 2 and 4 were plotted using Prism software (GraphPad Software, San Diego, CA). The lines for Figure 2A were calculated from the two phase exponential decay equation.

Results

The calibration and specificity of the ELISA

When the percentage competition by the antigen against the biotinylated probe was plotted against the amount of *Pya*-AKH, typical sigmoidal curves were obtained (Fig. 1 and Table 1). The range over which the assay could be used to measure *Pya*-AKH was between 20 - 1000 fmol per well of the microtitre plate.

To test the specificity of the ELISA, a range of other synthetic AKHs (and analogues) were used that were available in the laboratory. From the results of the ELISA, these fell arbitrarily into four groups: *Pya*-AKH, *Poa*-HrTH, the antigen Cys^{1}- *Pya*-AKH, the hormone analogue Asn^{7}-*Mem*-CC, *Pea*-CAH-II, and the pentapeptide QTPNWamide that were well-recognised by the IgG; *Pea*-CAH-I, *Tem*-HrTH, *Emp*-AKH, and *Mem*-CC that were moderately well-recognised; *Phm*-AKH, *Scd*-CC-I and II, *Pab*-RPCH, and *Lom*-AKH-III that were recognised only poorly; and *Lom*-AKH-I and II, *Grb*-AKH, *Scg*-AKH-II, *Pht*-HrTH that were not recognised at all at the highest concentrations of 20 pmol per well tested here (Table 1).

The amounts of the AKH in the CNS

When increasing concentrations of tissue extracts from the CNS of *Pyrrhocoris* were tested in the ELISA, even at the highest concentration tested, no *Pya*-AKH was detected in the brain or suboesophageal and ventral ganglionic mass (Fig. 2). Extracts of brain - corpora cardiaca/corpus allatum complexes did, however, contain sufficient amounts of material for measurement in the ELISA (Fig. 2). It was estimated that the corpora cardiaca/corpus allatum complex of one bug contained on average 3.8 ± 1.3 pmol of AKH (mean ± SE, n=5) that was extracted by the method employed here.

Measurement of Pya-AKH in the haemolymph

A number of attempts were made to detect endogenous AKH in simple methanolic extracts of haemolymph from bugs. But even in 100 µl of pooled haemolymph, no reliable determinations of the amounts of endogenous AKH could be made. To test whether the bug haemolymph contained material that interfered with measurement in the ELISA, bugs were injected with sufficiently large amounts of synthetic *Pya*-AKH, so that it should have been detectable in the ELISA. It can be seen from Fig. 3 that such exogenous *Pya*-AKH was detected in the haemolymph of bugs injected with as little as 10 pmol of hormone. Furthermore, it was clear that the amount of exogenous AKH that could be detected in the ELISA decreased rapidly with time. The half-life times during the first 40 min after injection were around 18 min, irrespective of the amount of exogenous AKH injected (Fig. 3).

When the detection of endogenous levels of AKH in simple methanolic extracts of haemolymph in the ELISA proved problematic, the extracts were further purified before testing in the ELISA. After solid phase extraction, HPLC separations generated a relatively small number of UV-absorbing peaks (Fig. 4A), which were tested in the ELISA for the presence of AKH. The results obtained from fractions in the region in which AKHs were expected to elute are shown in Fig. 4B. None of the other fractions were active (data not shown). The highest amount of AKH was detected in fraction 2, whose retention time was identical to that of synthetic *Pya*-AKH (retention time =6.87 min, dashed line in Figure 4A). The efficiency of recovery of AKH during the extraction procedure was checked by adding 500 fmol of *Pya*-AKH to 10-µl samples of haemolymph before beginning extraction. The recovery of AKH averaged 74.8 ± 8.2% (Mean ± SE, n=5). The level of AKH determined in haemolymph of the female bugs (Fig. 4B) after correction for losses was 1.14 ± 0.27 fmol/µl (Mean ± SE, n=3).

Discussion

The cross-reactivity studies show that the ELISA is quite specific. The competitive ability of the pentapeptide (5aa) suggests that, perhaps not surprisingly, the last four residues of *Pya*-AKH, TPNW, can account for much (but not all) of the recognition by the antibody. As a consequence, the IgG recognises *Pea*-CAH-II (only a change of threonine for asparagines at position 3) very well, which has important practical consequences (see below). However, it was initially surprising that *Lom*-AKH-I, for example, was not recognised by the IgG, and *Phm*-AKH was recognised only poorly: especially because *Pya*-AKH is des-[Gly^{9}, Thr^{10}]-*Lom*-AKH-I or des-[Gly^{9}, Ser^{10}]-*Phm*-AKH. It is most likely the absence of the tryptophan amide at the C-terminus that is responsible for this lack of recognition by the IgG. A previous report concerning the raising of antibodies to AKH molecules also suggests that the carboxiamide function is often recognised very well (Hekimi and O'Shea, 1989), so the spatial arrangement between the carboxiamide and the residues recognised is probably critical. Again, however, the poor recognition of *Lom*-AKH-III is surprising. It is clear that changes at some positions are critical, but a further change at a different position can sometimes show limited compensation. For example, valine in position 2 (*Emp*-AKH) is not favoured, but an additional change of serine instead of threonine at position 5 (*Pea*-CAH-I) improves recognition slightly. Overall, the data presented here suggest that Pro^{6}, Asn^{7} and Trp^{8} along with the C-terminal carboxiamide are the most important components of the epitope: three analogues that had tyrosine instead of Phe^{4} were recognized poorly but the data are insufficient to draw any firm conclusions about the importance of the phenylalanine residue in recognition by the IgG tested here.

The AKH-content of the corpora cardiaca/corpus allatum complex can be estimated by calibrating the hyperlipaemic response to tissue extracts against the dose-response data of Kodrík *et al*. (2000) for *Pya*-AKH in *Pyrrhocoris*. Thus, from that bioassay data, it seems likely that these glands could contain up to 10 pmol of *Pya*-AKH. The data presented here for the determination of the *Pya*-AKH-content using an ELISA, suggest a value of about 4 pmol per pair of corpora cardiaca. During the execution of this study a second AKH (identical to *Pea*-CAH-II) has been identified in the corpora cardiaca of *Pyrrhocoris* (Kodrík *et al*., 2002a); the present study shows that this second peptide is recognised only slightly less well than *Pya*-AKH in the ELISA. It may be that there are also other biologically active materials in the tissue extracts (perhaps octopamine and/or other biogenic amines) that would be recognised in a functional assay but not in the ELISA, so the values determined here may be realistic overall, but represent the sum of both AKHs present. Preliminary immunohistochemical evidence, using the IgG characterised here, shows that almost all of the immunoreactive material in *Pyrrhocoris* is contained within the corpora cardiaca, with just small amounts in a few cell groups and axons distributed over the whole brain (Zdenka Syrová, personal communication). AKH has also been identified in the brain of locusts (Moshitzky *et al*., 1987; Bray *et al*., 1993), but the physiological role of AKH-like immunoreactive material in the brain remains to be determined for any insect.

Such small amounts of AKH that are found in the bug corpora cardiaca are by no means unusual in those insects that have been studied in this respect, but are in marked contrast to the situation in a few other insects. For example, adult *Manduca sexta* corpora cardiaca contain about 10-30 pmol AKH (Ziegler, 1990; Fox and Reynolds, 1990), but this is much less than in locusts, where the values in newly emerged adults range from 100 - 200 pmol/corpora cardiaca and increase during adult

life (see Bray *et al*., 1993): maximal estimates for adults of *c*. 70 days old can be as high as 1,200 pmol (Siegert and Mordue, 1986; Oudejans *et al*., 1993). Because locusts fly for long periods, this could be a reflection of differences in the physiology of the insects. However, the highest reported content of AKH in the corpora cardiaca seems to be found, paradoxically, in a flightless grasshopper *Romalea microptera*, in which about 2,700 pmol of AKH are recorded in a single pair of corpora cardiaca from an adult male (Spring and Gäde, 1991).

Thus the amounts of AKH in the corpora cardiaca of *Pyrrhocoris* (3.8 pmol) are not abnormally low relative to the estimated blood volume (6 µl), and are consistent with the titres of AKH in the haemolymph shown by this study. Little information concerning the AKH content of insect haemolymph is available, perhaps due to the need for preliminary clean-up of the haemolymph and the large numbers of insects required, both to obtain sufficient volumes of pooled haemolymph and for use in bioassays. Cheeseman and Goldsworthy (1979) used a lipid mobilisation bioassay to estimate from pooled haemolymph that the level of AKH in the haemolymph of flown *Locusta* is between 1.6 and 4 fmol/µl, and more recently Candy (2002) has used a radioimmunoassay to determine the titres of AKH-I in the haemolymph of *Schistocerca* during rest and flight: values of 0.12 and between 1 and 3 fmol/µl respectively were obtained. Thus, although the titres of AKH determined here for *Pyrrhocoris* appear low, they are in fact comparable with the values determined in the haemolymph of locusts during flight. Indeed, they are considerably higher than the levels recorded in resting locusts. On the other hand, Santos and Keller (1993) claim that in the crab *Carcinus maenas*, basal levels of the crustacean hyperglycaemic hormone are around 0.02 fmol/µl and, according to Webster (1996), in the edible crab *Cancer pagurus* they are even lower (0.001 fmol/µl). The differences probably reflect the energy needs and thus the importance of the hormones in different kinds of locomotion: flight, walking and 'underwater' walking, respectively.

The difficulty in detecting appreciable levels of endogenous AKH in the haemolymph directly, could be due to the presence in the haemolymph of substances that interfere with the ELISA, or the existence of a bound pool of AKH in the haemolymph that is not readily extracted by methanol treatment. High levels of injected (exogenous) *Pya*-AKH are detected easily in the ELISA but, of course, this could be because any such interfering binding sites are saturated by the large amounts of hormone injected. Nevertheless, it is likely that to measure AKH in the haemolymph of *Pyrrhocoris* with any accuracy using the ELISA described here, large volumes of pooled haemolymph and preliminary cleanup (solid phase extraction and HPLC, for example) will be necessary. The need for extensive clean-up of haemolymph has been shown in locusts, in which the measurement of titres of AKH in haemolymph by (radio)immunoassay requires considerable pre-purification of the samples (Candy, 2002).

From the studies here, the turnover of the *Pya*-AKH in the haemolymph appears to be rapid with a half-life of *c*. 18 min. Half-lives in the order of a few minutes have also been proposed for AKHs from other insects. For example in locusts the endogenous AKHs are inactivated by cleavage of their Asn^3-Phe^4 bond (Siegert and Mordue, 1992) and their rates of degradation are different for each particular hormones and are influenced by locomotory activity: half-lives of AKH-I, -II and -III at rest are 51, 40 and 5 min; and 35, 37 and 3 min respectively during flight (Oudejans *et al*., 1996; Van der Horst *et al*., 1999).

Determination of the amounts of AKH in the corpora cardiaca (or in the CNS) and also in the haemolymph are important for the interpretation of the results of experiments designed to help understand the physiological role of AKH in the bug.

These experiments include a study of the release pattern of *Pya*-AKH from the corpora cardiaca into haemolymph, and the elucidation of the role of the hormone(s) in coordinating the hyperlipaemic and diel walking activity in macropterous females of *P. apterus* (Maxová *et al.*, 2001), or the elucidation of the role of stress in the expression of the complex response to adipokinetic hormones in the bug (Kodrík *et al.*, 2002b). These studies on the titres of AKHs in *Pyrrhocoris* are underway, and are the subject of a separate communication.

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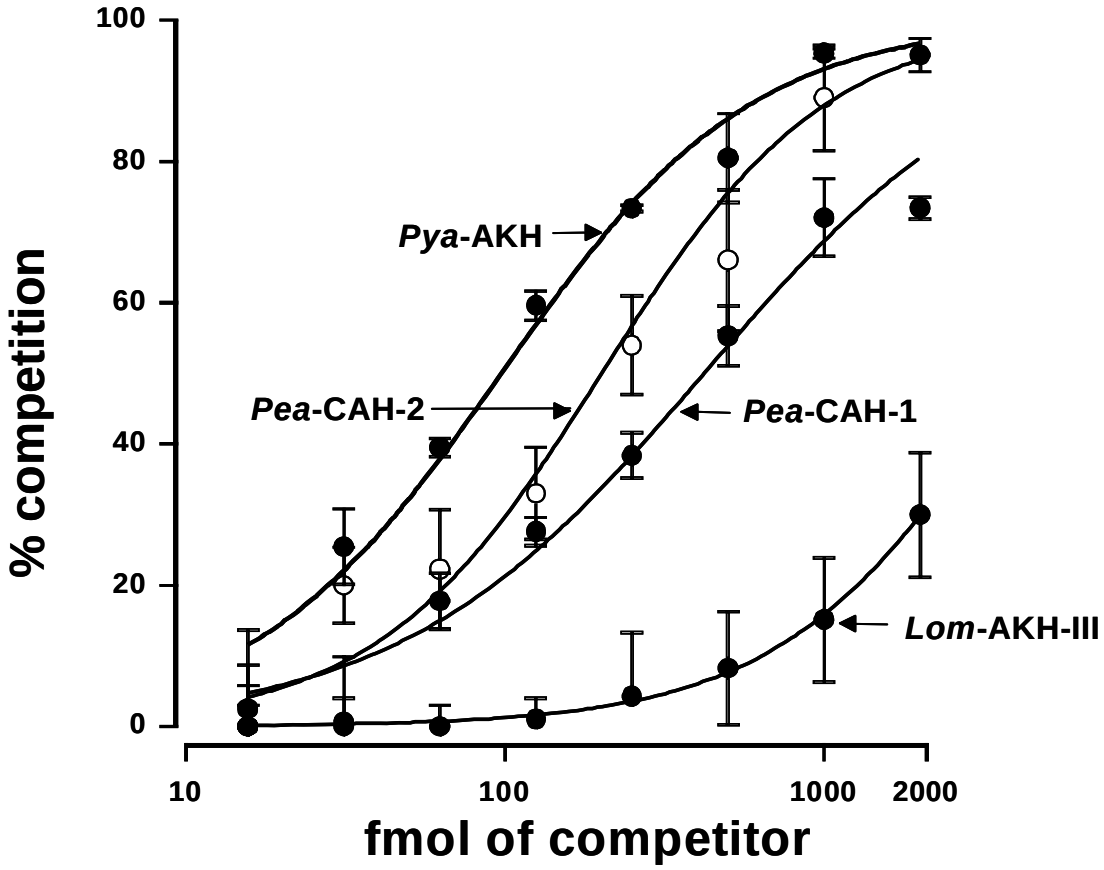

Fig 1. Competition curves for representative AKH peptides tested in the ELISA developed in this study. Each point represents the mean ± SE for 3 determinations.

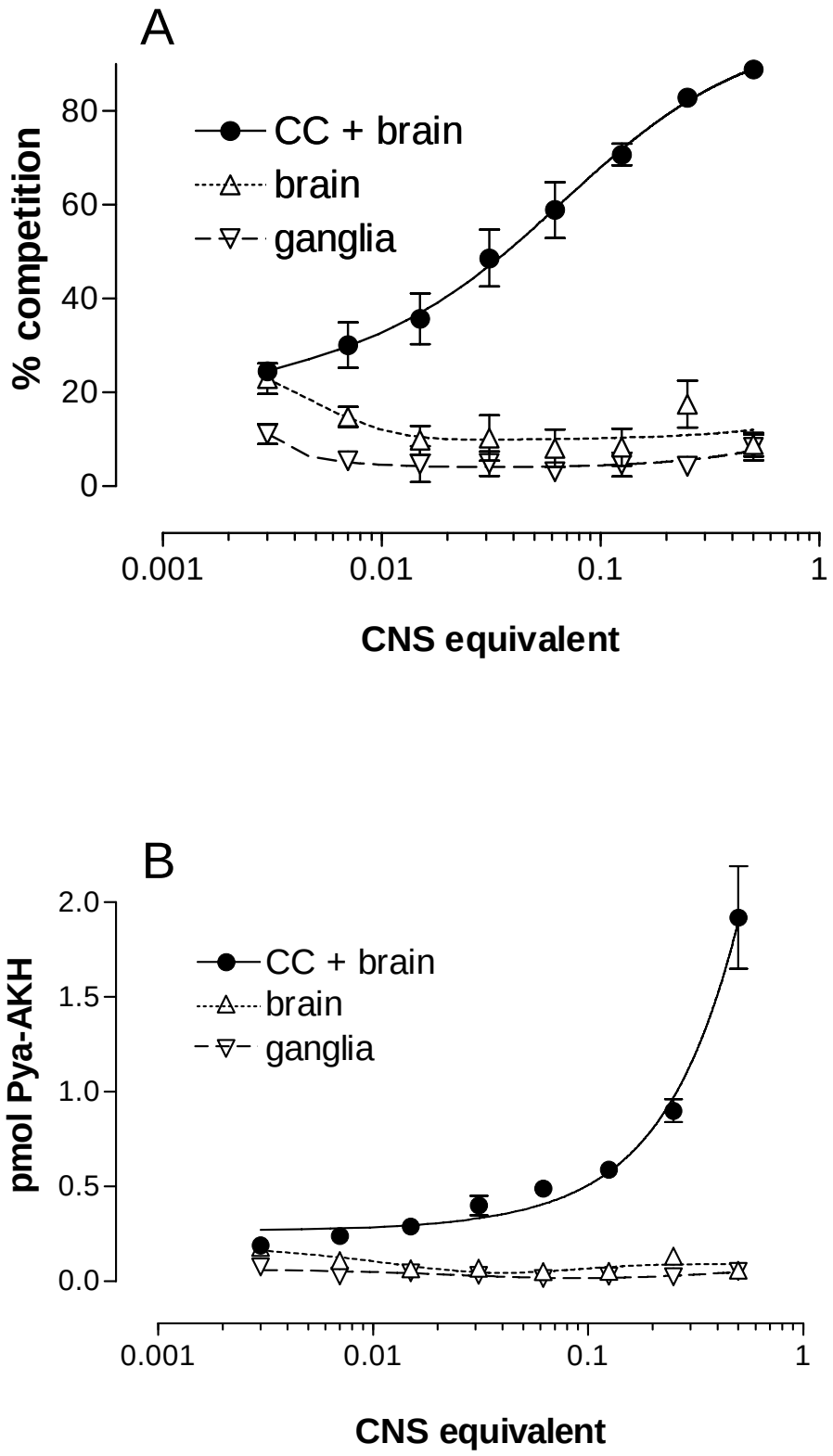

Fig. 2. (A) The effect of increasing doses of extracts of the neuroendocrine system of 7-10-day old *P. apterus* females (brain with corpora cardiaca, brain alone, and ganglionic mass: CNS equivalents) on increasing the percentage competition for AKH in the ELISA. (B) The same effect when the results are expressed as pmol AKH/CNS organs using synthetic *Pya*-AKH as a standard. Each point represents the mean ± SD for 5 determinations.

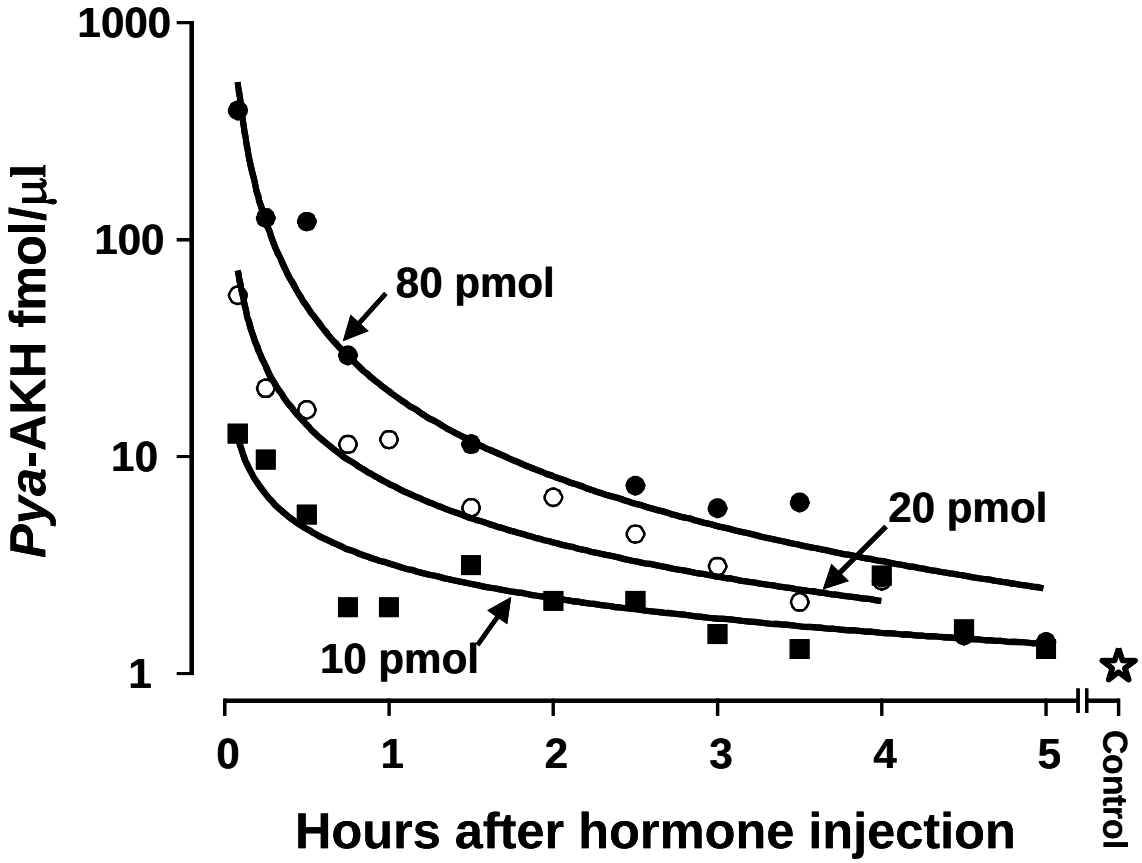

Fig. 3. Changes in the amount of AKH detected in the haemolymph of 10-day old female *P. apterus* injected with different amounts of *Pya*-AKH. The results represent the amount of AKH detected in 1 µl of haemolymph taken from a (single) different bug at each time point, and are expressed as fmol AKH/µl of haemolymph determined in the ELISA. The curves were fitted to the data in FigP (BioSoft) as power curves. Half lives of 17.5, 18.8, and 18.5 min were estimated from plots of logAKH titre against time for the first 45 min after injection of 80, 20 and 10 pmol of *Pya*-AKH respectively.

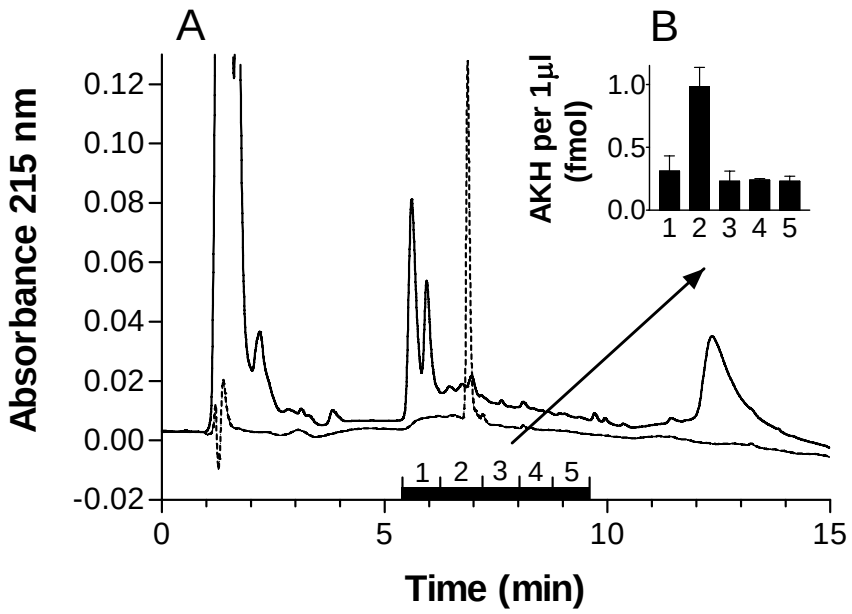

Fig. 4. (A) The RP HPLC elution profile of an extract of 10 µl of pre-purified haemolymph from 7-day old *P. apterus* females (solid line) and 1000 pmol synthetic *Pya*-AKH (dashed line, retention time =6.87 min). (B) Content of the endogenous AKH (vertical bars: mean ± SE, *n*=3) determined by the competitive ELISA in the haemolymph in fractions 1 - 5 (solid horizontal bar in Fig. 4A) expressed in fmol of AKH per 1 µl of haemolymph using synthetic *Pya*-AKH as a standard. For details see Materials and Methods.

Table 1.
The sequences of the peptides tested for their ability to compete with the biotinylated Cys[1]-*Pya*-AKH.Values for relative recognition >100 mean that the ability to compete is less than that for *Pya*-AKH.

Peptide name	Single letter code sequence	Relative recognition
Good recognition		
Poa-HrTH	QITFTPNWa	89
Pya-AKH	QLNFTPNWa	100
Cys[1]-*Pya*-AKH	CLNFTPNWa	110
Pea-CAH-II	QLTFTPNWa	210
Asn[7]-*Mem*-CC	QLNYSPNWa	210
5aa	QTPNWa	240
Moderate recognition		
Pea-CAH-I	QVNFSPNWa	380
Tem-HrTH	QLNFSPNWa	620
Emp-AKH	QVNFTPNWa	650
Mem-CC	QLNYSPDWa	970
Poor recognition		
Phm-AKH	QLNFTPNWGSa	1250
Scd-CC-II	QFNYSPVWa	1900
Scd-CC-I	QFNYSPDWa	2230
Pab-RPCH	QLNFSPGWa	2250
Lom-AKH-III	QLNFTPWWa	3800
No recognition		
Lom-AKH-I	QLNFTPNWGTa	-
Lom-AKH-II	QLNFSAGWa	-
Grb-AKH	QVNFSTGWa	-
Scg-AKH-II	QLNFSTGWa	-
Pht-HrTH	QLTFSPDWa	-